

University of Groningen

Purification and Characterization of Haloalcohol Dehalogenase from *Arthrobacter* sp. Strain AD2

van den Wijngaard, Arjen J.; Reuvekamp, Peter T.W.; Janssen, Dick B.

Published in:
Journal of Bacteriology

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
1991

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

van den Wijngaard, A. J., Reuvekamp, P. T. W., & Janssen, D. B. (1991). Purification and Characterization of Haloalcohol Dehalogenase from *Arthrobacter* sp. Strain AD2. *Journal of Bacteriology*, 173(1), 124-129.

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Purification and Characterization of Haloalcohol Dehalogenase from *Arthrobacter* sp. Strain AD2

ARJAN J. VAN DEN WIJNGAARD, PETER T. W. REUVEKAMP, AND DICK B. JANSSEN*

Department of Biochemistry, Groningen Biotechnology Center, University of Groningen, Nijenborgh 16, 9747 AG Groningen, The Netherlands

Received 4 June 1990/Accepted 25 September 1990

An enzyme capable of dehalogenating vicinal haloalcohols to their corresponding epoxides was purified from the 3-chloro-1,2-propanediol-utilizing bacterium *Arthrobacter* sp. strain AD2. The inducible haloalcohol dehalogenase converted 1,3-dichloro-2-propanol, 3-chloro-1,2-propanediol, 1-chloro-2-propanol, and their brominated analogs, 2-bromoethanol, as well as chloroacetone and 1,3-dichloroacetone. The enzyme possessed no activity for epichlorohydrin (3-chloro-1,2-epoxypropane) or 2,3-dichloro-1-propanol. The dehalogenase had a broad pH optimum at about 8.5 and a temperature optimum of 50°C. The enzyme followed Michaelis-Menten kinetics, and the K_m values for 1,3-dichloro-2-propanol and 3-chloro-1,2-propanediol were 8.5 and 48 mM, respectively. Chloroacetic acid was a competitive inhibitor, with a K_i of 0.50 mM. A subunit molecular mass of 29 kDa was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. With gel filtration, a molecular mass of 69 kDa was found, indicating that the native protein is a dimer. The amino acid composition and N-terminal amino acid sequence are given.

Both epichlorohydrin (3-chloro-1,2-epoxypropane) and its precursor in chemical synthesis, 1,3-dichloro-2-propanol, are industrial chemicals that may enter the environment because of their volatile character or improper disposal. These compounds are considered important environmental pollutants (12), in part because epichlorohydrin has been shown to be mutagenic and carcinogenic in rats (18).

Recently, three bacterial cultures able to grow on epichlorohydrin, 3-chloro-1,2-propanediol, or 1,3-dichloro-2-propanol were isolated (20). Two of these cultures, *Pseudomonas* sp. strain AD1 and *Arthrobacter* sp. strain AD2, were examined in more detail. Degradation of epichlorohydrin proceeded via 3-chloro-1,2-propanediol and glycidol (3-hydroxy-1,2-epoxypropane). *Arthrobacter* sp. strain AD2 was not capable of enzymatic opening of the epoxide ring in epichlorohydrin, but *Pseudomonas* sp. strain AD1 possessed an epoxide hydrolase, producing 3-chloro-1,2-propanediol.

It was shown that both strains dehalogenated haloalcohols by a highly inducible enzyme which we call haloalcohol dehalogenase (20). The dehalogenase catalyzed the conversion of vicinal haloalcohols to the corresponding epoxides with liberation of inorganic chloride or bromide. The same mechanism of dehalogenation of haloalcohols was found earlier in a *Flavobacterium* sp. isolated on 2,3-dibromo-1-propanol (1, 3).

The products of dehalogenation and the substrate range of the enzyme suggest that haloalcohol dehalogenases do not belong to other established classes of dehalogenases, such as haloacid dehalogenases (4, 15), haloalkane dehalogenases (6-8, 10, 16, 22), and dichloromethane dehalogenases (11, 17). Since no information on the biochemical characteristics of haloalcohol dehalogenases is available, we purified and studied the enzyme from *Arthrobacter* sp. strain AD2 in more detail. We also present evidence that the dehalogenase of *Pseudomonas* sp. strain AD1 differs from the dehalogenase of *Arthrobacter* sp. strain AD2.

MATERIALS AND METHODS

Organism and growth conditions. The organisms used in this study were *Arthrobacter* sp. strain AD2 (20), *Pseudomonas* sp. strain AD1 (20), *Pseudomonas* sp. strain 113 (14), *Pseudomonas* sp. strain GJ1 (9), *Xanthobacter autotrophicus* GJ10 (8), and the coryneform bacterial strain AD3 (20).

Arthrobacter sp. strain AD2 was grown in a 10-liter fermentor (Braun Biostat E, Melsungen, Federal Republic of Germany [FRG]) in a medium containing (per liter): 5.37 g of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 1.36 g of KH_2PO_4 , 1.25 g of $(\text{NH}_4)_2\text{SO}_4$, 0.4 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 ml of salts solution (9), and 20 mg of yeast extract (BBL Laboratories). Cells were grown at pH 7, 30°C, and 50% oxygen saturation under an air atmosphere. At different time points, with decreasing time intervals, 25-ml aliquots of a sterile 350 mM solution of 3-chloro-1,2-propanediol were supplied as a carbon source with a peristaltic pump. After the culture had reached a density of 0.7 mg (dry weight) per ml, cells were harvested in a continuous centrifuge (Sharples TIP).

Purification of the dehalogenase. Crude extracts were prepared as described earlier (20). The purification procedure used was almost the same as described previously for the isolation of the haloalkane dehalogenase of *X. autotrophicus* GJ10 (10). In short, 92 ml of crude extract containing 2.4 g of protein was fractionated by stepwise addition of solid ammonium sulfate. Precipitates were dissolved in 20 ml of TEM buffer (10 mM Tris sulfate, 1 mM EDTA, 1 mM β -mercaptoethanol [pH 7.5]) and assayed for activity, and the fractions with highest activity (45 to 50% and 50 to 60% precipitates) were pooled and dialyzed for 11 h against 3 liters of this buffer. The dialyzed material was applied to a DEAE-cellulose column (30 by 2 cm) that was previously equilibrated with TEM buffer. After the column was washed with 20 ml of this buffer, elution was carried out with a linear gradient of 0 to 0.5 M ammonium sulfate in TEM buffer (total volume, 500 ml; flow rate, 25 ml/h; fraction volume, 7.5 ml). Active fractions, found at 80 to 160 mM ammonium sulfate, were pooled and dialyzed overnight against 5 liters of potassium phosphate buffer (5 mM, pH 6.6). The dialyzed fraction was applied to a hydroxylapatite column (Bio-Rad

* Corresponding author.

HTP, 13 by 0.7 cm) that was previously equilibrated with potassium phosphate buffer (5 mM, pH 6.6). The dehalogenase passed through the column without retention (flow rate, 25 ml/h; fraction volume, 7.5 ml). The five most active fractions were pooled, and the dehalogenase was concentrated by ammonium sulfate precipitation (80% saturation) and dissolved in 3 ml of TEM buffer. Further purification took place by gel filtration with a Sephacryl S-200 column (80 by 2 cm) that was previously equilibrated with TEM buffer (flow rate, 10 ml/h; fraction volume, 5 ml). The two most active fractions were pooled and frozen at -20°C until use.

Biochemical characterization. The molecular mass of denatured dehalogenase was estimated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) by the method of Laemmli (13). Ovotransferrin (78 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), and myoglobin (17 kDa) were used as marker proteins. The gel contained 10% acrylamide and was stained with Coomassie brilliant blue.

The molecular mass of the native enzyme was determined by gel filtration with a Sephadex G-100 column (80 by 2 cm) that was previously equilibrated with TEM buffer (flow rate, 5 ml/h; fraction volume, 4.1 ml). Reference proteins (10 mg each) were bovine serum albumin, ovalbumin, trypsin inhibitor (20 kDa), and cytochrome *c* (12.3 kDa). The molecular mass was also estimated by fast protein liquid chromatography (FPLC; Pharmacia) with a Superose 12 gel filtration column that was previously equilibrated with sodium phosphate buffer (50 mM, pH 7) supplemented with 0.1 M ammonium sulfate. A sample (1.6 mg) of purified enzyme in 1 ml of TEM buffer was dialyzed overnight against 2 liters of demineralized water (4°C) and subjected to freeze drying, after which the sample was dissolved in 1 ml of sodium phosphate buffer (50 mM, pH 7) containing 0.1 M ammonium sulfate. Then, 200 μl was applied to the column, and 0.5-ml fractions (flow rate, 0.5 ml/min) were collected. Molecular mass standards were alcohol dehydrogenase (150 kDa), bovine serum albumin, ovalbumin, trypsin inhibitor, and cytochrome *c*.

For amino acid analysis, a fraction of purified enzyme was dialyzed against demineralized water and subsequently hydrolyzed for 24 h with 6 N HCl at 110°C . The hydrolysates were analyzed with a Kontron amino acid analyzer.

The N-terminal amino acid sequence of the protein was determined with a gas phase protein sequencer (Applied Biosystems model 477A), with on-line phenylthiohydantoin-amino acid identification (ABI model 120A) (5) by Euro Sequence, Groningen.

Localization of dehalogenase on polyacrylamide gels. Dehalogenase was visualized after electrophoresis on 10% polyacrylamide slab gels by the method of Weightman and Slater (21). The buffers used contained 1 mM β -mercaptoethanol and 1 mM EDTA, and the gel was prerun overnight at 4°C . Samples of purified enzyme (40 μg of protein) were mixed with electrophoresis buffer (21) and loaded in preformed wells in the stacking gel. Electrophoresis was carried out at 4°C and 30 mA for 5 h. The gel was cut in several parts. One part was stained with Coomassie brilliant blue, while other parts were incubated for 30 min with 5 mM 1,3-dichloro-2-propanol in 50 mM Tris-sulfate buffer (pH 7.5) at room temperature. After incubation, the gel parts were washed with deionized water and placed in a solution of 0.1 M AgNO_3 . Formation of AgCl precipitation bands indicated the regions of dehalogenase activity. In subsequent gels, proteins other than the dehalogenase did not produce precipitation bands in this way.

Dehalogenase assays. Dehalogenase assays were carried out by monitoring halide release from halogenated substrates as described before (20). Solutions of halogenated compounds were freshly made before each assay. Of all halogenated compounds used, only 1-bromo-2-propanol, 1,3-dibromo-2-propanol, and 1,3-dichloroacetone showed nonenzymatic halide production under the assay conditions (20). For the calculation of enzyme activities with these three substrates, nonenzymatic halide production was subtracted from total halide production as described earlier (20). For routine assays, 1,3-dichloro-2-propanol was used as the substrate. One unit of enzyme activity is defined as the activity that catalyzed the formation of 1 μmol of halide per min under the conditions used.

Protein was measured by the method of Bradford (2a) with bovine serum albumin as the standard.

Assays for identification of the products formed during dehalogenation were done as described before (20).

Kinetic constants were determined by measuring initial velocities of halide production at different substrate concentrations.

Halogenation and transhalogenation. The halogenation and transhalogenation of several compounds, such as epichlorohydrin and epibromohydrin (3-bromo-1,2-epoxypropane), were monitored by gas chromatography (GC). Incubation mixes (6 ml) contained 5 mM substrate, 0.1 M KBr or NaCl, and 100 μl of purified enzyme. After 0, 5, 30, and 120 min of incubation at 30°C , samples (1.5 ml) were removed and extracted with diethylether containing 1 mM trichloroethylene as an internal standard. Ether extracts were analyzed by GC on a CPWax52-CB column (Chrompack) with the procedure and equipment described previously (20).

Immunochemical analysis. Antiserum against the haloalcohol dehalogenase of *Arthrobacter* sp. strain AD2 was raised by injecting a rabbit intramuscularly with 250 μg of purified dehalogenase in complete Freund adjuvant. Three subsequent injections of purified dehalogenase in complete Freund adjuvant were administered at 10-day intervals. Ten days after the last injection, blood was collected and the serum containing the polyclonal antibodies was separated from the blood cells by centrifugation (10 min, $8,000 \times g$).

Western immunoblot analysis with peroxidase-conjugated antibody was performed essentially as described previously (19; see also the Bio-Rad instruction manual). The gel and the nitrocellulose sheet (Schleicher & Schull, 0.45- μm pore size) were sandwiched between 4 (under) and 5 (over) sheets of 3MM Whatman paper in transfer buffer (25 mM Tris hydrochloride [Tris-HCl], 150 mM glycine, 20% [vol/vol] methanol, pH 8.3) and placed in an electroblot apparatus (Pharmacia) that was filled with transfer buffer. Electrophoresis was performed for 2 h at 0.35 to 0.40 A and 4°C . Further steps were performed at room temperature, with gentle shaking. The nitrocellulose sheet was blocked by washing 8 times for 10 min in TTBS buffer (20 mM Tris-HCl, 200 mM NaCl, 0.05% Tween 20 [pH 7.5]). The primary-antibody incubation mix (60 min) contained a 1:1,000 dilution of antiserum in TTBS buffer. The nitrocellulose sheet was subsequently washed 6 times for 10 min with TTBS buffer. The secondary-antibody treatment (60 min) was performed with a 1:5,000 dilution of horseradish peroxidase coupled to goat anti-rabbit immunoglobulin (Bio-Rad Laboratories) in TTBS buffer. After the nitrocellulose sheet was washed 6 times for 10 min in TTBS buffer and two times for 10 min in TBS buffer (20 mM Tris-HCl, 200 mM NaCl [pH 7.5]), the antigens were visualized as described in the Bio-Rad instruction manual.

TABLE 1. Purification of haloalcohol dehalogenase

Fraction	Total protein (mg)	Total activity (U)	Sp act (U/mg of protein)	Yield (%)	Purification (fold)
Crude extract	2,438	550	0.23	100	
Ammonium sulfate	1,116	494	0.44	90	2.0
DEAE-cellulose	365	457	1.25	83	5.5
Hydroxylapatite	68	193	2.83	35	12.5
Sephacryl S-200	16.5	58	3.52	10	15.7

GC-MS. All halogenated compounds and other organic substrates, such as glycidol, propylene oxide, 1,2-propanediol, hydroxyacetone, and ethylene glycol, were analyzed by injecting water samples into a GC-mass spectrometer (MS; Ribermag R 10-10c) as described earlier (20).

Chemicals. All organic compounds used were obtained from commercial sources (20).

RESULTS

Purification of the haloalcohol dehalogenase. The purification scheme of the haloalcohol dehalogenase of *Arthrobacter* sp. strain AD2 is given in Table 1. Gel electrophoresis under denaturing conditions (SDS-PAGE) indicated that the protein preparation obtained after gel filtration was more than 95% pure (Fig. 1). By activity staining of nondenaturing gels, it was found that the protein band comigrated with the protein showing enzyme activity and thus represented the dehalogenase (Fig. 2). The enzyme was purified 16-fold, with an overall yield of 10%. This implies that the enzyme represents about 5 to 6% of the total soluble cellular protein of *Arthrobacter* sp. strain AD2, assuming that the purified enzyme retains the specific activity of the protein in crude extracts. The dehalogenase was the major protein present in crude extracts when the organism was grown on 3-chloro-1,2-propanediol (Fig. 1).

We found that the purified dehalogenase was very stable. The enzyme could be stored in 10 mM Tris buffer (pH 7.5)

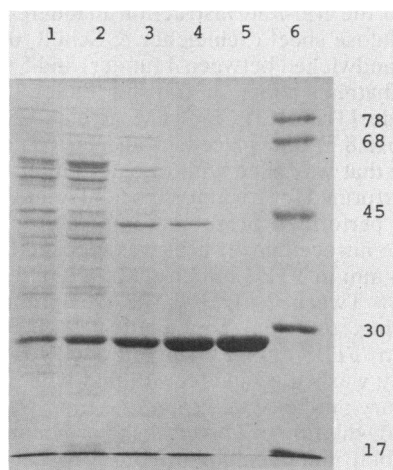


FIG. 1. SDS-PAGE of haloalcohol dehalogenase of *Arthrobacter* sp. strain AD2. Lanes: 1, crude extract; 2, 45 to 60% ammonium sulfate precipitate; 3, DEAE-cellulose step; 4, hydroxylapatite step; 5, Sephacryl S-200 step; 6, molecular mass standards (shown in kilodaltons). Lanes each contained 20 μ g of protein.

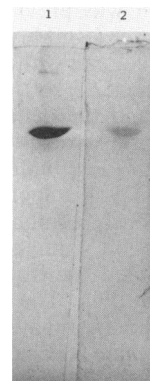


FIG. 2. Nondenaturing gel electrophoresis of purified haloalcohol dehalogenase of *Arthrobacter* sp. strain AD2. Lanes: 1, stained with Coomassie brilliant blue; 2, activity stain (see Materials and Methods). Each lane contained 40 μ g of protein.

containing 1 mM β -mercaptoethanol and 1 mM EDTA at -20 or 4°C for 6 months without significant loss of activity.

Molecular mass. The molecular mass of the dehalogenase was estimated to be 29 kDa by SDS-PAGE (Fig. 1). Molecular weights of 69,000 and 65,000 were found with gel filtration of the native enzyme on a Sephadex G-100 column and on a Superose 12 column (FPLC), respectively, indicating that the dehalogenase is a dimeric protein, consisting of two monomers that have an identical electrophoretic mobility.

Substrate range. The haloalcohol dehalogenase converted C_2 and C_3 haloalcohols and catalyzed liberation of halide from vicinal mono- and dichlorinated alcohols as well as

TABLE 2. Substrate specificity of the haloalcohol dehalogenase

Activity and substrate	Rate ^a (% of control)	Product
Dehalogenation^b		
1,3-Dichloro-2-propanol	100	Epichlorohydrin
1-Chloro-2-propanol	11	Propylene oxide
3-Chloro-1,2-propanediol	10	Glycidol
1,3-Dichloroacetone	356	ND ^c
Chloroacetone	10	Hydroxyacetone
1,3-Dibromo-2-propanol	31,100	Epibromohydrin
1-Bromo-2-propanol	3,780	Propylene oxide
2-Bromoethanol	102	Ethylene oxide
Halogenation		
Epichlorohydrin + NaCl	47	1,3-Dichloro-2-propanol
Epichlorohydrin + KBr	161	1-Bromo-3-chloro-2-propanol
Epibromohydrin + NaCl	11	1-Bromo-3-chloro-2-propanol
Epibromohydrin + KBr	117	1,3-Dibromo-2-propanol
Propylene oxide + KBr	22	1-Bromo-2-propanol
Transhalogenation		
Epichlorohydrin + KBr	1.6	Epibromohydrin
Epibromohydrin + NaCl	63	Epichlorohydrin

^a A rate of 100% corresponds to 2,037 mU/mg of protein, which was found with 1,3-dichloro-2-propanol as the substrate.

^b No halide liberation was found with epichlorohydrin, 2-chloroacetic acid, 2-chloroethanol, 2-chloropropionic acid, 3-chloropropionic acid, 1,3-dichloropropane, 1,3-dichloropropene, 1-chloropropane, 2-chloropropane, 1,2-dichloropropane, 1,2-dichloroethane, 1,2-dibromopropane, 1-bromo-3-propanol, 2,3-dichloro-1-propanol, and 2-chlorophenol.

^c ND, Not determined.

TABLE 3. Effect of potential inhibitors and substrate analogs on enzyme activity

Reagent ^a	Concn (mM)	Inhibition (%)
EDTA	1	0
HgCl ₂	1	66
Iodoacetic acid	1	62
N-Ethylmaleide	1	0
p-Chloromercuribenzoate	0.001	12
1-Chloropropane	5	0
1,2-Dichloropropane	5	0
1,2-Dichloroethane	5	0
Epichlorohydrin	5	0
Chloroacetic acid	5	87
2-Chloropropionic acid	5	33
3-Chloropropionic acid	5	92

^a The enzyme was incubated for 5 min at 30°C with the compound indicated before dehalogenase activity was determined with 1,3-dichloro-2-propanol as the substrate.

mono- and dibrominated alcohols (Table 2). In all cases, halide production rates were much higher with brominated substrates than with their chlorinated analogs. Of the chlorinated alcohols tested, 1,3-dichloro-2-propanol was the best substrate.

With several substrates, the products were identified by GC-MS (Table 2). 1,3-Dichloro-2-propanol was dehalogenated to epichlorohydrin, and 1-chloro-2-propanol and 2-bromoethanol were converted to propylene oxide and ethylene oxide, respectively. Bromopropanols were also converted to epoxides. None of the epoxides were further degraded by the dehalogenase. These results show that dehalogenation of haloalcohols, catalyzed by the purified enzyme, occurred with formation of epoxides, as previously proposed on the basis of identification of products in crude extracts (20).

The dehalogenase was also active with chloroacetone and 1,3-dichloroacetone. Chloroacetone was converted to hydroxyacetone by the purified enzyme. No haloalcohol dehalogenase activity was found with haloalkanes and 2-halocarboxylic acids. Moreover, no activity was detected with 2,3-dichloro-1-propanol.

The reverse reaction of dehalogenation of haloalcohols was tested with several epoxides in the presence of 0.1 M halide. Bromination and chlorination of epichlorohydrin and epibromohydrin were observed (Table 2). Bromination of propylene oxide (Table 2) and 1,2-epoxyoctane (data not shown) was also detected.

The transhalogenation reactions (Table 2) are probably the result of the combination of halogenation and dehalogenation.

Enzyme kinetics. The dehalogenase showed a broad pH optimum around 8.5. The temperature optimum was 50°C. Above this temperature, the enzyme rapidly became inactivated. From the temperature versus activity curve, an activation energy of 49.4 kJ/mol was calculated for the conversion of 1,3-dichloro-2-propanol.

With 1,3-dichloro-2-propanol as the substrate, it was found that the enzyme followed Michaelis-Menten kinetics. By measuring the initial velocities of chloride release at different substrate concentrations, a K_m of 8.5 mM and V_{max} of 9.0 μ mol/min/mg of protein were found. The K_m for 3-chloro-1,2-propanediol was 48 mM and the V_{max} was 3.1 μ mol/min/mg of protein.

Inhibitors. Several substrate analogs and possible inhibi-

TABLE 4. Amino acid composition of the haloalcohol dehalogenase

Amino acid	Content (mol%)	No. of residues ^a
Aspartic acid	9.9	26.1
Threonine	5.8	15.3
Serine	6.6	17.4
Glutamic acid	8.0	21.1
Proline	8.0	21.1
Glycine	8.9	23.5
Alanine	15.9	41.9
Valine	3.2	8.4
Methionine	1.2	3.2
Isoleucine	3.4	9.0
Leucine	8.7	22.9
Tyrosine	2.7	7.1
Phenylalanine	6.0	15.8
Lysine	2.0	5.3
Histidine	1.3	3.4
Arginine	8.4	22.1
Cysteine	0	0

^a Values were calculated on the basis of a molecular weight of 29,000.

tors were tested with the purified protein (Table 3). Thiol reagents such as HgCl₂ and p-chloromercuribenzoate inhibited the enzyme only partly, whereas N-ethylmaleimide did not inhibit the enzyme at all under these conditions.

Inhibition of the dehalogenase was found with haloalkanoic acids (Table 3). Chloroacetic acid turned out to be a competitive inhibitor, with a K_i of 0.50 mM.

Amino acid composition and N terminus. The amino acid analysis of the dehalogenase showed alanine as the most abundant amino acid (Table 4). Other predominant amino acids were glycine, leucine, and arginine. No cysteine or cystine residues were found.

The first 34 residues of the N terminus were sequenced (Fig. 3). The sequence does not show any remarkable features except that alanine was found seven times. A search of the SWISS.PROT protein sequence data base release 13.0 did not yield homologous sequences.

Immunochemical relationship with other dehalogenases. To test whether the dehalogenase of strain AD2 is related to other bacterial dehalogenases, crude extracts from different strains were analyzed by immunoblotting with antiserum against the purified haloalcohol dehalogenase of *Arthrobacter* sp. strain AD2. The halocarboxylic acid dehalogenase of *Pseudomonas* sp. strain 113 has a similar molecular mass (two 30-kDa subunits) and an amino acid composition that closely resembles that of the dehalogenase described here. Extracts prepared from cells of *Pseudomonas* sp. strain 113 grown on 2-chloropropionic acid contained 596 mU of halo-carboxylic acid dehalogenase per mg of protein but possessed no activity for 1,3-dichloro-2-propanol or 1-bromo-2-propanol. As shown by immunoblotting (Fig. 4), this bacterium also produced no protein that is immunochemically related to the dehalogenase of strain AD2. The same was found with a crude extract made of cells of *Pseudomo-*

```

1      5      10      15
MET-LYS-ILE-ALA-LEU-VAL-THR-HIS-ALA-ARG-HIS-PHE-ALA-GLY-PRO
20      25      30
ALA-ALA-VAL-GLU-ALA-LEU-THR-ARG-ASP-GLY-TYR-THR-VAL-VAL-X-
34
HIS-ASP-ALA-SER-

```

FIG. 3. N-terminal amino acid sequence of the haloalcohol dehalogenase. X, Unidentified residue.

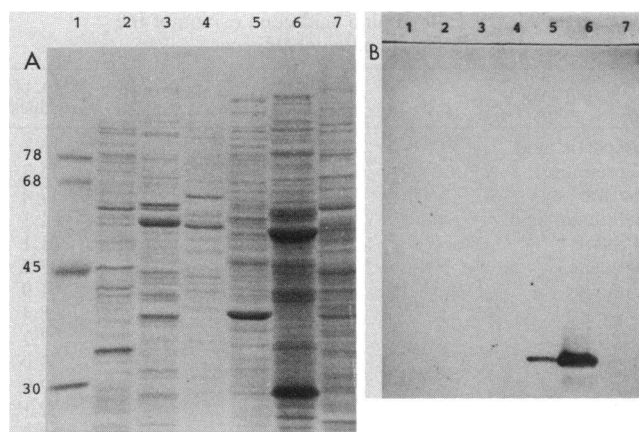


FIG. 4. SDS-PAGE (A) and Western blot analysis (B) of crude extracts prepared from six different bacterial cultures. The panels represent identical gels that were run at the same time. Antiserum prepared against the purified dehalogenase of *Arthrobacter* sp. strain AD2 was used. Lanes: 1, markers; 2, *Pseudomonas* sp. strain 113 grown on 2-chloropropionic acid; 3, *X. autotrophicus* GJ10 grown on 1,2-dichloroethane; 4, strain GJ1 grown on 2-chloroethanol; 5, strain AD3 grown on epichlorohydrin; 6, *Arthrobacter* sp. strain AD2 grown on epichlorohydrin; 7, *Pseudomonas* sp. strain AD1 grown on epichlorohydrin. Lanes contained 20 to 40 μ g of protein.

nas sp. strain GJ1 grown on 2-chloroethanol and of *X. autotrophicus* GJ10 grown on 1,2-dichloroethane.

Pseudomonas sp. strain AD1 was found to possess haloalcohol dehalogenase activity when grown on epichlorohydrin (20). In contrast to the dehalogenase of strain AD2, however, this enzyme showed no reaction with antiserum against the dehalogenase of *Arthrobacter* sp. strain AD2 (Fig. 4), suggesting that it is different from the dehalogenase of strain AD2.

The epichlorohydrin-degrading gram-positive organism strain AD3 (20), grown on epichlorohydrin, also showed haloalcohol dehalogenase activity. In crude extracts, activity was detected with 1,3-dichloro-1-propanol (175 mU/mg of protein), 1-bromo-2-propanol (3,437 mU), and 2-bromoethanol (75 mU). These activities closely follow the relative activities of the dehalogenase purified from *Arthrobacter* sp. strain AD2, as shown in Table 2. After Western blotting, extracts of strain AD3 showed an immunochemically stained band with the same electrophoretic mobility as the dehalogenase of strain AD2 (Fig. 4).

DISCUSSION

Carbon-halogen bond cleavage is a critical step in the microbial utilization of halogenated aliphatic compounds. Different reaction mechanisms have been identified, and several of the dehalogenating enzymes have been characterized. These include glutathione transferases catalyzing nucleophilic displacement of halogen substituents of dihalomethanes (11, 17) and hydrolytic dehalogenases that show activity with 2-halocarboxylic acids (14, 15) and haloalkanes (6, 10, 16, 22). In this report, we present for the first time data on the biochemical characteristics of an enzyme that catalyzes dehalogenation of vicinal haloalcohols through intramolecular displacement with formation of epoxides. As a systematic name, we propose haloalcohol hydrogen-halide-lyase (EC 4.5.1).

The dehalogenase studied is the key enzyme of haloalcohol metabolism in *Arthrobacter* sp. strain AD2 (20). It was isolated to more than 95% purity from cells grown in a fed batch culture with 3-chloro-1,2-propanediol as the carbon source. The purified dehalogenase possessed activity towards 2-bromoethanol and a variety of C_3 chloroalcohols, C_3 bromoalcohols, and C_3 chloroketones. Among the substrates are various environmentally important compounds or intermediates in the degradation of xenobiotic pollutants, including epichlorohydrin, 1,3-dichloro-2-propanol, and 2-bromoethanol. The rates observed with bromoalcohols were about 100-fold higher than the rates with the corresponding chlorinated compounds. The products formed from haloalcohols were epoxides. For this reaction, the water-soluble dehalogenase did not require cofactors or oxygen. Thus, the mechanism of dehalogenation is likely to proceed via intramolecular substitution. The poor utilization of 1,3-dihalo-2-propanols as a growth substrate (20) may be explained by the absence of epoxide hydrolase activity in strain AD2. Together with the high dehalogenase activity for 1,3-dibromo-2-propanol, this could lead to toxicity of this compound as a result of epibromohydrin accumulation.

The same type of dehalogenation as described here was found with a partially purified haloalcohol epoxidase from a *Flavobacterium* sp. that was isolated on 2,3-dibromo-1-propanol (1). The *Flavobacterium* enzyme dehalogenated C_3 haloalcohols, such as 2,3-dibromo-1-propanol, with concomitant formation of epoxides, but it possessed no activity towards halogenated C_3 ketones (3), as we found for the *Arthrobacter* dehalogenase. Moreover, the *Flavobacterium* enzyme did not convert 3-chloro-1,2-propanediol or 3-bromo-1,2-propanediol to glycidol (3).

The haloalcohol dehalogenase of *Arthrobacter* sp. strain AD2 appeared to be a dimer consisting of two 29-kDa polypeptides. Several hydrolytic dehalogenases were also found to be water-soluble enzymes of relatively low molecular weight, including halocarboxylic acid dehalogenases (4, 15) and haloalkane dehalogenases (6, 10, 16, 22).

Haloalkane dehalogenases isolated from *X. autotrophicus* GJ10 (10), *Arthrobacter* sp. strain HA1 (16), and strain GJ70 (6) catalyze the hydrolytic dehalogenation of haloalkanes, haloalcohols, and haloethers to the corresponding alcohols. So far, 2-bromoethanol is the only substrate converted by both these enzymes and the haloalcohol dehalogenase described here. The hydrolytic conversion of 2-bromoethanol by purified haloalkane dehalogenase from *X. autotrophicus* GJ10 resulted in the formation of ethylene glycol at 1.4 U/mg of protein (A. J. van den Wijngaard and K. W. H. J. van der Kamp, unpublished results), while the haloalcohol dehalogenase of strain AD2 converted 2-bromoethanol to ethylene oxide (3.5 U/mg of protein with pure enzyme), showing that this compound may be dehalogenated by two different mechanisms.

Immunoblotting experiments were performed in order to test whether the haloalcohol dehalogenase from AD2 was related to representatives of hydrolytic dehalogenases. Crude extracts of *X. autotrophicus* GJ10 containing both a haloalkane dehalogenase and a halocarboxylic acid dehalogenase did not react with antiserum against the purified dehalogenase of strain AD2, indicating that the enzymes probably have no strong amino acid sequence homology. The halocarboxylic acid dehalogenases of *Pseudomonas* sp. strain GJ1 and *Pseudomonas* sp. strain 113 also were found not to be immunologically related, although the latter enzyme has a similar amino acid composition and its substrate

chloroacetic acid was a competitive inhibitor of the AD2 haloalcohol dehalogenase.

Immunological cross-reactivity was also tested with other organisms producing haloalcohol dehalogenases. Crude extracts of the coryneform bacterial strain AD3 (20) gave a reaction with antibodies raised against strain AD2 haloalcohol dehalogenase. Strain AD3 grown on epichlorohydrin possessed dehalogenase activity with several haloalcohols, and on SDS-polyacrylamide gels a 29-kDa band reacting with the antibodies was detected, indicating that its dehalogenase is similar to the AD2 enzyme. With crude extracts of *Pseudomonas* sp. strain AD1, on the other hand, no immunochemical relationship with the AD2 dehalogenase was found. Together with different substrate profiles (20), this clearly indicates that this organism produces a haloalcohol dehalogenase that is distinct from the enzyme of strain AD2. Since the *Flavobacterium* enzyme also has different characteristics, as mentioned above, the class of haloalcohol dehalogenases may contain at least three different types of enzymes.

The dehalogenase content of *Arthrobacter* sp. strain AD2 grown on 3-chloro-1,2-propanediol was 5 to 6%. With cells grown on epichlorohydrin, the specific activity in crude extracts was 3 to 4 times higher (870 mU/mg of protein) (20), meaning that the dehalogenase content of strain AD2 can be increased to approximately 18 to 22%. This high level of a dehalogenase is not unusual in bacteria degrading xenobiotic compounds. From 12 to 20% of the protein content of the dichloromethane-utilizing bacterium DM2 is a highly inducible dehalogenase (11). The function of the high dehalogenase content of *Arthrobacter* sp. strain AD2 could be to compensate for its low turnover and specificity or first-order rate constant with some growth substrates (e.g., $k_{\text{cat}} = 3.0 \text{ s}^{-1}$ and $k_{\text{cat}}/K_m = 0.06 \text{ s}^{-1} \text{ mM}^{-1}$ with 3-chloro-1,2-propanediol), thus allowing cell growth at lower substrate levels. Strains with a higher dehalogenase content possibly have a selective advantage if dehalogenation is the growth rate-limiting step.

ACKNOWLEDGMENTS

We thank Albert Kiewiet for the GC-MS analyses, Peter Jekel for performing the amino acid analysis, Janny de Wit for determination of the molecular weight by FPLC, and Bernard Witholt for reading the manuscript. Thanks are due to K. Soda for a gift of *Pseudomonas* sp. strain 113.

This work was financed in part by a grant from the Dutch Ministry of Housing, Physical Planning and Environment. The work of D.B.J. was made possible by a fellowship from the Royal Academy of Sciences of the Netherlands.

REFERENCES

1. Bartnicki, E. W., and C. E. Castro. 1969. Biodehalogenation. The pathway for transhalogenation and the stereochemistry of epoxide formation from haloalcohols. *Biochemistry* 8:4677-4680.
2. Bergmann, J. G., and J. Sanik. 1957. Determination of trace amounts of chlorine in naphtha. *Anal. Chem.* 29:241-243.
- 2a. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.
3. Castro, C. E., and E. W. Bartnicki. 1968. Biodehalogenation. Epoxidation of haloalcohols, epoxide opening, and transhalogenation by a *Flavobacterium* sp. *Biochemistry* 7:3213-3218.
4. Hardman, D. J., and J. H. Slater. 1981. Dehalogenases in soil bacteria. *J. Gen. Microbiol.* 123:117-128.
5. Hewick, R. M., M. W. Hunkapiller, L. E. Hood, and W. J. Dreyer. 1981. A gas-liquid solid phase peptide and protein sequencer. *J. Biol. Chem.* 256:7990-7997.
6. Janssen, D. B., J. Gerritse, J. Brackman, C. Kalk, D. Jager, and B. Witholt. 1988. Purification and characterization of a bacterial dehalogenase with activity towards halogenated alkanes, alcohols and ethers. *Eur. J. Biochem.* 171:67-72.
7. Janssen, D. B., D. Jager, and B. Witholt. 1987. Degradation of *n*-haloalkanes and α,ω -dihaloalkanes by wild type and mutants of *Acinetobacter* sp. strain GJ70. *Appl. Environ. Microbiol.* 53:561-566.
8. Janssen, D. B., A. Scheper, L. Dijkhuizen, and B. Witholt. 1985. Degradation of halogenated aliphatic compounds by *Xanthobacter autotrophicus* GJ10. *Appl. Environ. Microbiol.* 49:673-677.
9. Janssen, D. B., A. Scheper, and B. Witholt. 1984. Biodegradation of 2-chloroethanol and 1,2-dichloroethane by pure bacterial cultures. *Progr. Ind. Microbiol.* 20:169-178.
10. Keuning, S., D. B. Janssen, and B. Witholt. 1985. Purification and characterization of hydrolytic haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10. *J. Bacteriol.* 163:635-639.
11. Kohler-Staub, D., and T. Leisinger. 1985. Dichloromethane dehalogenase of *Hyphomicrobium* sp. strain DM2. *J. Bacteriol.* 162:676-681.
12. Krijgheld, K. R., and A. van der Gen. 1986. Assessment of the impact of the emission of certain organochlorine compounds on the aquatic environment. Part 3: epichlorohydrin. *Chemosphere* 15:881-893.
13. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* 227:680-685.
14. Motosugi, K., N. Esaki, and K. Soda. 1982. Purification and properties of a new enzyme, *dl*-2-haloacid dehalogenase, from *Pseudomonas* sp. *J. Bacteriol.* 150:522-527.
15. Motosugi, K., and K. Soda. 1983. Microbial degradation of synthetic organochlorine compounds. *Experientia* 39:1214-1220.
16. Scholtz, R., T. Leisinger, F. Suter, and A. M. Cook. 1987. Characterization of 1-chlorohexane halohydrinase, a dehalogenase of wide substrate range from an *Arthrobacter* sp. *J. Bacteriol.* 169:5016-5021.
17. Scholtz, R., L. P. Wackett, C. Egli, A. M. Cook, and T. Leisinger. 1988. Dichloromethane dehalogenase with improved catalytic activity isolated from a fast-growing dichloromethane-utilizing bacterium. *J. Bacteriol.* 170:5698-5704.
18. Sram, R. J., L. Tomatis, J. Clemmesen, and B. A. Bridges. 1981. An evaluation of the genetic toxicity of epichlorohydrin. *Mutat. Res.* 87:299-319.
19. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* 76:4350-4354.
20. van den Wijngaard, A. J., D. B. Janssen, and B. Witholt. 1989. Degradation of epichlorohydrin and haloalcohols by bacterial cultures isolated from freshwater sediment. *J. Gen. Microbiol.* 135:2199-2208.
21. Weightman, A. J., and J. H. Slater. 1980. Selection of *Pseudomonas putida* strains with elevated dehalogenase activities by continuous culture growth on chlorinated alkanoids. *J. Gen. Microbiol.* 121:187-193.
22. Yokota, T., T. Omori, and T. Kodama. 1987. Purification and properties of haloalkane dehalogenase from *Corynebacterium* sp. strain m15-3. *J. Bacteriol.* 169:4049-4054.